

# BUFOTALIN PROMOTES TRAIL-INDUCED APOPTOSIS IN NON-SMALL LUNG CANCER CELLS

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**Abstract**— This study investigates the effects of bufotalin, a bufodienolides compound isolated from toad venom, on sensitizing TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of non-small lung cancer cell lines, A549 and PC-9. Cells were treated with bufotalin in the presence and absence of TRAIL. The cells viability was observed using WST-1(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) assay. The TRAIL-induced apoptotic signaling proteins were examined by immunoblotting. The results showed that TRAIL alone showed potent cytotoxicity on PC-9 cells, but weakly induced the death of the A549 cells, indicating that A549 cells are a TRAIL-resistance cell line and PC-9 cells are a TRAIL-sensitive cell line. Bufotalin significantly enhanced death receptor 4-(DR4) induced cell death in the A549 cell line through the activation of caspase-3 and PARP-1. The combination of bufotalin with TRAIL resulted in the down-regulation of Bid, c-FLIP, and the up-regulation of DR4, RB and p53. In contrast, TRAIL dramatically induced cell death and bufotalin has no effect on TRAIL-induced PC-9 cell death. The results indicate that bufotalin sensitizes the A549 cells, but not the PC-9 cells to TRAIL-induced apoptosis through DR4-induced apoptotic pathway.

**Index Terms**— TRAIL, Bufotalin, DR4, Bid, apoptosis.

## I. INTRODUCTION

Apoptosis or programmed cell death is the process in which a cell self-degrades to eliminate dysfunctional cells. The deregulation of apoptosis results in diseases, including autoimmune, cancer, and neurodegenerative disorders [1]. Apoptosis can be divided into extrinsic pathway and intrinsic pathway. The intrinsic pathway is triggered by the releasing of signal factors from mitochondria within the cell. The extrinsic pathway is activated through the stimulation of the death receptors, such as the TNF-related apoptosis-inducing ligand (TRAIL) receptors [2-5]. TRAIL induces apoptosis in various human cancer cell lines, but they do not affect normal cells. It is expected that compounds targeting these death receptors will be designed to lower toxicity and increase antitumor activity [6-8]. However, many cancers are resistant to TRAIL-based therapies mainly due to the decreased expression of death receptors and up-regulation of TRAIL pathway-mediated anti-apoptotic proteins. The molecules that regulate TRAIL signaling in cell death are also involved in the resistance. Low expression of death receptors and p53 have been found to cause resistance to TRAIL [9-11]. In lung, head and neck cancer cells, mutation of DR4 affected TRAIL binding to DR4. Cell-survival proteins, such as the Bcl-2-family protein, Bid have been reported to play major roles in TRAIL resistance. A contribution of c-FLIP to TRAIL resistance has been detected in various types of cancer cells [12-13]. Compounds that reverts such defects restore the sensitivity of cancer cells to TRAIL, suggesting that combined

therapies could help manage neoplastic patients. Bufotalin (Fig.1A), one of the bufadienolides isolated from Formosan Ch'an Su, which is a widely used traditional Chinese medicine (TCM), exhibited strong anticancer, cardiotonic and anesthetic activities [14]. It has been reported that bufotalin naturally target the lung and brain cells. This may be a promising antitumor candidate for lung cancer [15]. Non-small lung cancer cell is the most common type of lung cancer. A549 cell line is a gefitinib resistant-carcinomic human alveolar basal epithelial cell, which consists of mutated K-ras, wild type EGFR and PC-9, a EGFR mutant lung cancer cell line which is sensitive to gefitinib. This study investigated the effects of bufotalin on TRAIL-induced apoptosis in two different non-small lung cancer cells, The A549 and PC-9 cell lines.

## II. MATERIAL AND METHODS

### A. Cell culture and proliferation assay

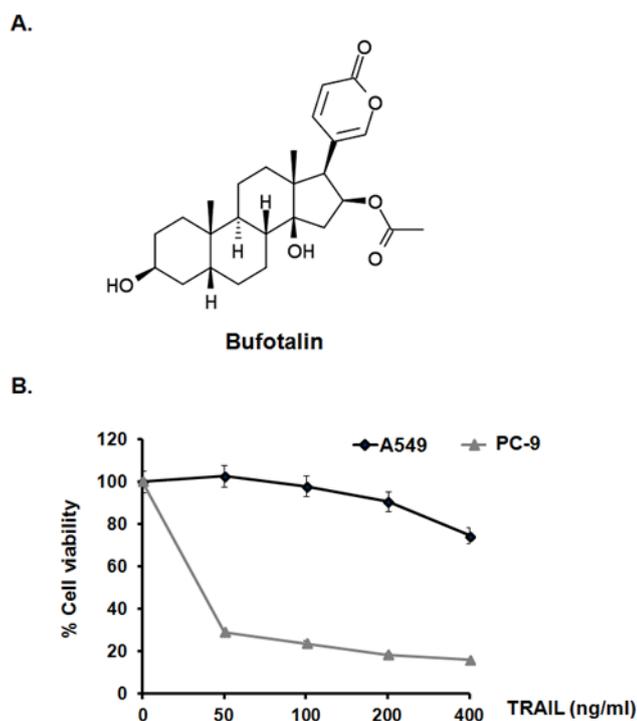
A549 and PC-9 cells were cultured in RPMI-1640 supplemented with 100 units/ml penicillin, 10% fetal bovine serum and 100 µg/ml streptomycin, and then incubated at 37°C in 5% CO<sub>2</sub>. The cell viability was quantified by using the cell proliferation reagent WST-1 (DOJINDO, Kumamoto, Japan). The cells were plated in 96-well microplates at 6x10<sup>3</sup> cells/wells, and then incubated for 24 hours. Bufotalin containing medium in various concentrations were added to the wells, and the cells were incubated for 30 minutes, then stimulated with TRAIL 200 ng/ml. After a 24 hours-incubation period, 10 µl of WST-1 solution were added, and absorbance was measured at the wavelength 450 nm.

### B. Preparation of cell extracts

Cells were treated with 0.1 µM bufotalin, with or without 200 ng/ml TRAIL, and whole cell lysate was prepared by adding lysis buffer (25 mM HEPES pH 7.7, 0.3 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 10% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 µg/ml aprotinin, and 10 µg/ml leupeptin). The cell lysate was collected from supernatant after centrifugation at 14,000 rpm for 10 minutes.

### C. Immunoblotting

Cell lysate was resolved by SDS-PAGE and transferred to a PVDF-membrane (Millipore). The membrane was treated with BlockAce (Dainippon Pharmaceutical Co. Ltd, Suita, Japan) and probed with primary antibodies. The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit and anti-goat IgG (DAKO, Glostrup, Denmark), and visualized with the enhanced chemiluminescence system (Amersham Biosciences).



**Figure 1. TRAIL induced apoptosis of A549 and PC-9 cells** A. Chemical structure of bufotalin. B. A549 and PC-9 cells were treated with TRAIL in various concentrations for 24. Cell proliferation was determined by MTT assay.

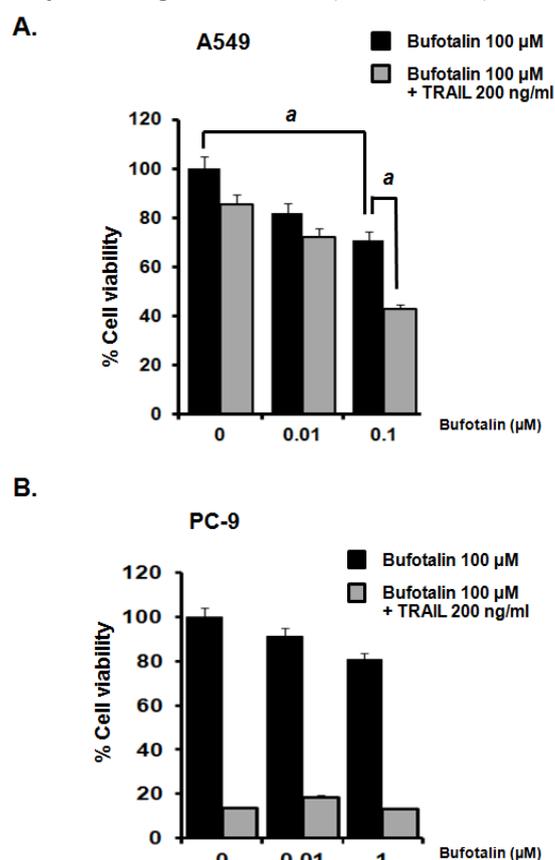
### III. RESULTS

#### A. Cytotoxic effect of TRAIL on lung cancer cells

To test the effect of TRAIL on cell viability, A549 and PC-9 lung cancer cells were treated with TRAIL in various concentrations for 24 hours and the cell viability was determined by using WST-1 assay. **Fig.1B** shows that TRAIL revealed weak cytotoxicity in A549 cells, but strongly decreased viability of PC-9 cells. This result indicates that A549 is a TRAIL-resistant cell line and the PC-9 cell is sensitive to TRAIL-induced cell death.

#### B. Bufotalin sensitized TRAIL-induced lung cancer cell death

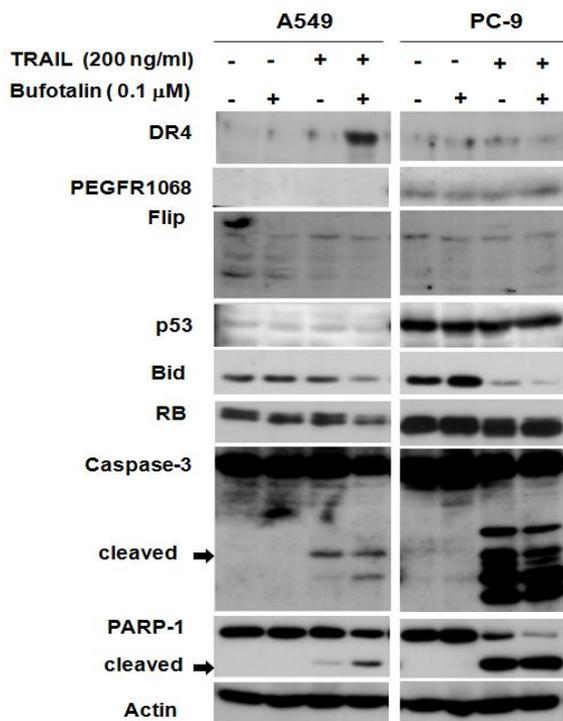
To investigate the effect of bufotalin on TRAIL-resistant and TRAIL-sensitive lung cancer cells, A549 and PC-9 cells were treated with bufotalin, with or without TRAIL. In A549 cells, bufotalin showed cytotoxicity to cancer cells and significantly enhanced TRAIL induced cell death at concentration 0.1  $\mu\text{M}$  (**Fig. 2A**). In contrast, bufotalin did not affect to TRAIL-induced cell death, and TRAIL alone dramatically induced PC-9 cell death (**Fig. 2B**). These result indicate that bufotalin sensitize A549 cells (TRAIL-resistant cell line) to TRAIL.



**Figure 2. Effect of bufotalin on TRAIL-induced A549 and PC-9 cell death.** A. A549 and PC-9 cells. B. were pretreated with 100  $\mu\text{M}$  bufotalin for 30 minutes and treated with TRAIL for 24 hours. Cell viability was investigated by MTT assay.  $^*P < 0.01$ .

#### C. Effect of bufotalin on TRAIL-induced DR4 mediated apoptotic signaling pathway in A549 and PC-9 lung cancer cells

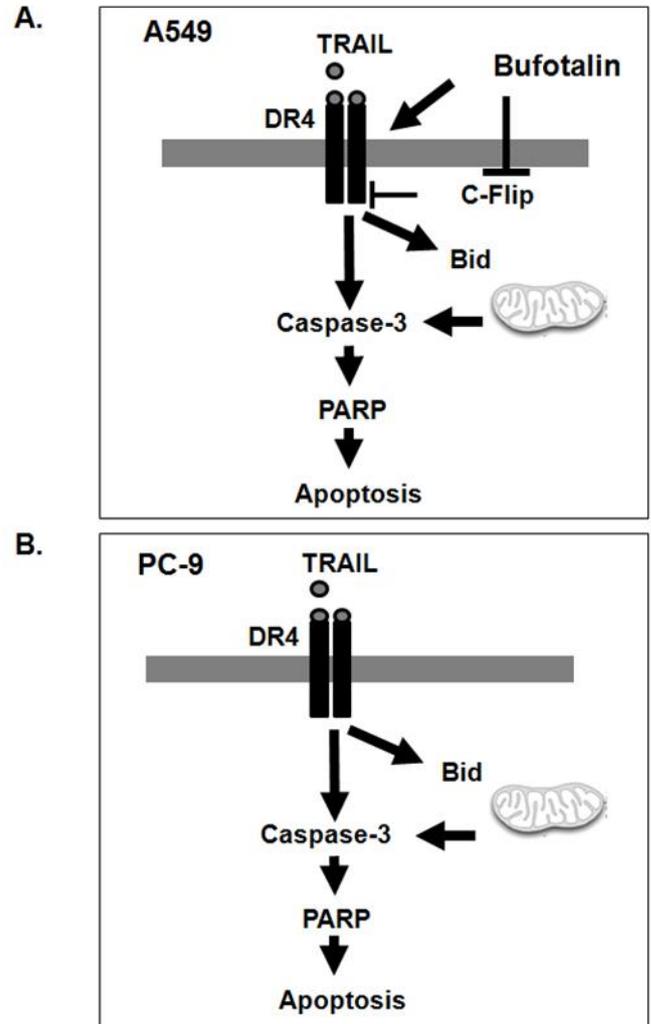
In order to explain the mechanism by which bufotalin sensitizes A549 cells to TRAIL, we investigated the effect of bufotalin on TRAIL-induced caspase-3 and PARP-1 activation as a hallmark of apoptotic response. In addition, apoptotic signaling proteins, including phospho-EGFR, DR4, p53, c-FLIP and Bid were analyzed by using western blot. The result revealed that bufotalin triggered TRAIL-induced cleave of caspase-3 and PARP-1 through DR4 up-regulation, Bid activation and decreased c-FLIP in A549 cells. In PC-9 cells, TRAIL, but not bufotalin induced caspase-3 and PARP-1 activation through Bid down-regulation (**Fig. 3**). The result indicates that bufotalin sensitize resistant cells to TRAIL via DR4 activation.



#### IV. DISCUSSION

Death receptors are cell-surface receptors belonging to the tumor necrosis factor (TNF) superfamily that includes TRAIL receptors (DR4 and DR5). Among the six different death receptors (DRs) identified to date, DR4 and DR5 are selectively expressed on cancer cells [5, 10]. TRAIL induces apoptosis mainly through DR-mediated pathways. Binding of TRAIL to DR4 and DR5 enables the receptors to homotrimerize, which leads to activation of caspase-8, Bid and caspase-3 subsequent to apoptosis. Many chemotherapeutic agents induce tumor cell death through TRAIL-mediated death receptors, such as wogonin and zerumbone overcome TRAIL-related apoptosis, by down-regulation of c-FLIP protein and up-regulation of TRAIL receptor expression [16-19]. Bufotalin, a bufadienolides isolated from Formosan Chan Su, has been used as an important TCM for heart failure and pains. Chan Su has been known to display antitumor activities [20]. Bufadienolides have antitumor activities through its ability to induce apoptosis in many cancer cells, including breast cancer, prostate cancer, lung cancer, hepatocellular carcinoma, gastric cancer and leukemia [21]. Previous reports have found that bufadienolides promotes TRAIL-mediated death receptors-induced apoptosis via STAT3/Mcl-1 and STAT1/DR5 pathways [22-23]. This study investigates the effect of bufotalin on the two different cell lines: A549, a TRAIL-resistant cell line and TRAIL-sensitized cell line and PC-9 cell lines. We found that bufotalin sensitized A549 cells to TRAIL through up-regulation of DR4, down-regulation of c-FLIP, activation of Bid and caspase-3 (Fig. 4A). Compared to A549, PC-9 cells showed a higher level of phospho-EGFR, p53 and RB, only TRAIL strongly induced Bid down-regulation and activation of caspase-3 and PARP-1 (Fig. 4B), but bufotalin was unaffected on this pathway. This finding suggests that bufotalin enhanced TRAIL-induced apoptosis of A549 cells via DR4 pathway.

**Figure 3. Effect of bufotalin on TRAIL-induced apoptotic regulating proteins in lung cancer cells.** Cells were treated with bufotalin in the presence or absence of TRAIL for 4 hours. Whole cell extract was prepared and analyzed by Western blotting using anti-phospho-EGFR, DR4, p53, RB, Bid, caspase-3, PARP-1 and β-actin antibodies. Arrows indicate cleaved forms of caspase-3 and PARP-1.



**Figure 4. Mechanism of bufotalin on TRAIL-induced apoptotic pathways in non-small lung cancer cells.**

A. Bufotalin sensitized A549 cell to TRAIL via DR4 up-regulation, c-FLIP inhibition and activation of caspase-3. B. PC-9, a TRAIL sensitive cell line, was activated by TRAIL via Bid and caspase-3 pathway, which resulted in apoptosis.

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